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In the preliminary studies conducted in our laboratory, administration of deprenyl, a monoamine oxidase-B (MAO-B) inhibitor, enhanced immunological functions [in vitro interleukin-2 (IL-2) production and NK cell activity], reversed the age-related loss of noradrenergic fibers in the spleens of old male rats, and prevented the development of carcinogen-induced mammary tumors in young female rats. The focus of the present study was to investigate whether treatment of young Sprague-Dawley female rats with deprenyl would inhibit the development and growth of 9, 10-dimethyl-1,2-benzanthracene- (DMBA-) induced mammary tumors by augmenting T-cell functions. Female Sprague-Dawley rats (50- to 55-day old) were administered DMBA orally. After the development of tumors, the rats were assigned to various groups and treated intraperitoneally with saline, 0.25 mg, 2.5 mg, or 5.0 mg of deprenyl/kg BW daily for 13 weeks. At the end of the treatment period, there was a significant reduction in tumor growth and tumor number in rats that received 2.5 mg and 5.0 mg/kg deprenyl. There also was a significant increase in splenic NK-cell activity in rats that received 2.5 mg and 5.0 mg of deprenyl. These results suggest that the administration of deprenyl blocked the development and growth of mammary tumors in part by improving immune reactivity.

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David L. Felten 12/24/97

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Introduction

Prolactin (PRL) from the anterior pituitary and estrogen from the ovaries are the two important hormones that influence mammary tumorigenesis; they act alone or synergistically to promote development and growth of mammary tumors. Neurotransmitters and neuropeptides from the hypothalamus have been reported to either inhibit (dopamine, norepinephrine, acetylcholine) or stimulate (serotonin, opioids, vasoactive intestinal peptide) PRL secretion and thus, exert an indirect control over mammary tumor growth (1). Treatment of rats with agents that increase PRL secretion promotes the growth while agents that decrease PRL secretion inhibit the growth, of both spontaneous and carcinogen-induced mammary tumors (2-4). The age-related decline in hypothalamic dopaminergic activity, especially the tuberoinfundibular dopaminergic (TIDA) system, has been shown to promote spontaneous development of pituitary and mammary tumors (1).

Recently, it was demonstrated that treatment of adult acyclic rats and carcinogen-induced mammary tumor rats with deprenyl reduced the incidence of tumors and inhibited the tumor growth, respectively (5). Deprenyl is a selective and potent inhibitor of monoamine oxidase-B (MAO-B) (6). In combination with levodopa, deprenyl therapy has been shown to delay the progression of Parkinson's disease through an enhancement in the neurotransmission of nigrostriatal dopaminergic pathway (7). In addition, deprenyl increased dopaminergic activity and inhibited DA re-uptake in the striatum, which are believed to be responsible for the increase in sexual activity and lifespan in rats (8).

In an earlier study, we found that prolonged treatment of old male F344 rats and young sympathectomized male rats with deprenyl caused restoration and acceleration, respectively, of sympathetic NA fibers into the splenic white pulp and increased splenic natural killer (NK) cell activity and IL-2 production in old male rats (9, 10). Rats with carcinogen-induced and spontaneously occurring mammary tumors have altered immunocompetence (11-13). The objective of this study was to investigate whether administration of deprenyl to rats with carcinogen-induced mammary tumors would inhibit the development and growth of tumors accompanied by improvement in cell-mediated immune functions.

Materials and Methods

Animals: Female Sprague-Dawley rats (40- to 42-day-old) were purchased from Charles River Laboratories, Kingston, NY and housed (5 animals/cage) in a temperature-controlled and light-controlled (12:12 h light/dark cycle) animal room. All animals received food and water ad libitum.

Treatment: After 10 days of acclimatization, each animal was administered with a single dose of 10 mg of 9, 10-dimethyl-1,2-benzanthracene (DMBA;Sigma, St. Louis, MO) dissolved in 1 ml of peanut oil by gastric intubation and housed individually. A separate group of rats (n=8) received 1 ml of peanut oil alone. Most of the rats developed mammry tumors within 3 months after DMBA administration. After tumor appearance, the rats were randomly divided into four different groups.

Rats in group 1 were injected with saline (n=12) which was used as the vehicle for injection of deprenyl (R(-)-Deprenyl hydrochloride; RBI, Natick, MA) to animals in groups 2 to 4. Rats in groups 2 (n=4), 3 (n=10), and 4 (n=13) were injected with 0.25. 2.5, and 5.0 mg of deprenyl/kg bw/day. All the injections were intraperitoneally for 13 weeks. Tumor diameter and tumor number were measured every week throughout the treatment period. Tumor diameter was calculated by averaging two perpendicular diameters determined by vernier calipers.

At the end of the treatment period, animals were decapitated, spleens were removed aseptically and cut into four equal blocks. One of the four blocks of spleen was used for immunological assays including NK cell activity, Con A-induced proliferation of lymphocytes and flow cytometry..

Lymphocyte preparation: A block of the spleen was placed into Hanks' balanced salt solution (HBSS; Sigma) containing sodium bicarbonate and HEPES (United States Biochemical

Corp., Cleveland, OH). The tissues were then dissociated using a Stomacher Lab-Blender (Tekmar Co., Cincinnati, OH). Cell suspensions were passed through fine nylon mesh to remove large aggregates and washed one time in HBSS. Erythrocytes were removed by layering the cell suspension on Histopaque 1077 (Sigma, St. Louis, MO), and centrifuging for 30 min at 2500 rpm. Cells were removed from the interface between the HBSS and the Histopaque and washed three times in HBSS. After the final wash, cells were resuspended to the desired concentration in RPMI 1640 medium supplemented with 5% fetal calf serum (Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.01 mM nonessential aminoacids, 5 $x10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 24 mM sodium bicarbonate, and 10 mM HEPES for in vitro culture.

NK cell activity: NK cell activity was assessed using the NK-sensitive lymphoma YAC-1 passaged in vitro. YAC-1 cells in log phase growth were incubated with 100 µCi of Na_2 $^{51}CrO_4$ (DuPont NEN, Boston, MA) at 37°C for 90 min. The cells were washed three times and adjusted to 10^5 cells/ml. Spleen cells ratios were mixed with 10^4 ^{51}Cr-labeled YAC-1 cells at varying effector to target (E:T) in round-bottom 96-well tissue culture plates (Falcon, Becton Dickinson) in triplicate in a volume of 200 µl. Spontaneous release was determined by incubating 10^4 ^{51}Cr-labeled YAC-1 cells with complete RPMI alone. Maximum release was determined by adding 1% Triton X-100 to 10^4 ^{51}Cr-labeled YAC-1 cells. The plates were centrifuged at 200 *g* for 5 min and incubated for 4 h at 37°C in a CO_2-humidified atmosphere. The plates were centrifuged at 500 *g* for 5 min at 4°C, and 100 µl of supernatant was removed from each well, and radioactivity was counted in a gamma counter. Cytotoxic activity was expressed as percent lysis, determined by the equation (experimental cpm-spontaneous cpm)/(maximum cpm-spontaneous cpm) X 100.

Con A-induced proliferation of lymphocytes: Spleen cells, 2 X 10^5 cells/ml, were cultured in triplicate with either medium alone or varying concentrations of Con A (Calbiochem-Behring Corp., La Jolla, CA) in 96-well, flat bottom tissue culture plates (Falcon), and maintained for 3 days at 37°C in a humidified 5% CO_2 incubator. [^{3}H]Thymidine (0.5 µCi/10µl; 5Ci/mmol; DuPont NEN, Boston, MA) was added for the final 18 h culture. Cells were harvested on to glass fiber filters (Whatman Inc., Clifton, NJ) with a cell harvester (Skatron, Sterling, VA). The dried filters were placed in scintillation fluid (Biosafe II; RPI, Mount Prospect, IL), and radioactivity determined with a liquid scintillation counter (LKB, Wallac, Finland).

Flow cytometric analysis: Spleen cells were washed in PBS containing 2% BSA and 0.02% azide (flow wash). Fluorescin-conjugated anti-rat sIgM (clone G53-238 diluted 1:40; Pharmingen, San Diego, CA) and phycoerythrin-conjugated anti-NK (clone NK1.1 (is this right?)diluted 1:40; Pharmingen) were added to 2 X 10^6 cells and incubated at 4°C for 30 min. To another tube, fluorescin-conjugated anti-rat CD8 (1:40) and phycoerythrin-conjugated anti-CD4 (1:20) were added to 2 X 10^6 cells and incubated at 4°C for 30 min. Cells incubated with flow-wash alone were included as a control for autofluorescence. Following this incubation, cells were washed twice in flow-wash, fixed in PBS containing 1% paraformaldehyde, and stored in the dark for no longer than 2 weeks at 4°C prior to analysis. Two-color fluorescence was analyzed with an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL), equipped with an argon-laser at 15 mW and excitation wavelength of 488 nM.

Statistical analysis: The data were analyzed by ANOVA. Mitogen (Con A) concentration and E:T ratio were treated as repeated measures. Parameters that attained significance following ANOVA were further analyzed by Fisher's least significant difference test.

Results

Tumor diameter: Tumor growth during the 13-week treatment period is shown in Figure 1.During the 13-week treatment period, the tumor diameter increased by 93.3±14.2 %

(mean±SE) in the saline group and by 63.8±26.2 % in the Dep 0.25 group. In contrast treatment with 2.5 mg and 5.0 mg of deprenyl significatly ($P<0.05$) decreased the tumor size by 6.5±14.6 % and 6.1±15.2 %, respectively, at the end of the treatment period.

Tumor Number: Treatment of rats with saline resulted in a gradual increase in the average number of tumors from the beginning to the end of the treatment period (Figure 2). Similar increases in the tumor number were observed in rats that received 0.25 mg of deprenyl. This increase in tumor number, however, was abrogated ($P<0.05$) by treatment with 2.5 mg and 5.0 mg of deprenyl from week 11 to 13.

NK cell activity: NK cell activity was significantly enhanced in rats that were treated with 2.5 mg and 5.0 mg deprenyl at E:T ratio, 80:1 and 40:1 (Figure 3).

Con A-induced proliferation of lymphocytes: Figure 4 shows that in vitro Con A-stimulated proliferation of spleen cells from saine- and deprenyl-treated rats was reduced at 1.25 µg of Con A/ml in comparison to rats that received oil alone. The proliferative capacity of spleen cells was lower in saline group at 5.0 µg/ml than that of oil group.

Flow cytometry: Deprenyl treatment of rats with carcinogen-induced mammary tumors did not alter the populations of sIgM+ B cells, NK+ cells, and CD4+ and CD8+ Th1 cells. There was a reduction in the population of CD8+ T cells in rats that received DMBA in comparison to rats that received oil.

Discussion:

We have demonstrated that deprenyl inhibits the development and growth of carcinogen-induced mammary tumors. Higher doses of deprenyl was more effective than the lower dose of deprenyl in blocking the increase in tumor growth and tumor burden. These effects of deprenyl on tumors were accompanied by an increase in NK cell activity.

In an earlier study, we have demonstrated that deprenyl inhibits the incidence and development of DMBA-induced mammary tumors in rats matched by a decrease in the metabolism of dopamine, norepinephrine, and serotonin in the medial basal hypothalamus (5). The inhibition of DA metabolism by deprenyl is significant because of the regulation of PRL secretion by dopamine in the anterior pituitary (14). The release of dopamine from the tuberoinfundibular dopaminergic neuronal system in the medial basal hypothalamus is dependent upon the activity of MAO because of lack of high-affinity transporter system and autoreceptors for dopamine (15-18). Several studies have reported that development and growth of mammary tumors are positively correlated with the systemic availability of PRL and that any treatment procedures that negatively interfered with this availability suppressed the growth of the spontaneous and carcinogen-induced mammary tumors in rats (2-4). Other studies have demonstrated that both acute and chronic administration of deprenyl inhibited PRL secretion in young and old female rats, and inhibited the incidence of mammary and pituitary tumors in old female rats (19-21).

A number of studies have demonstrated that the immune functions are suppressed in rats with carcinogen-induced and spontaneously occurring mammary tumors (11-13). In vitro studies have revealed that there is a suppression of mitogens-induced proliferation of thymocytes and splenocytes, inhibition of IL-2 receptor expression, and a reduction in the number of thymocytes and splenocytes isolated from rats with DMBA-induced mammary tumors (22). Several new methods are employed to reverse this immunosuppression, such as introduction of human IL-2 gene into the tumor to combat mammary tumorigenesis (23). Natural killer cells are one among many lymphoid cell subsets that are implicated in the non-specific immunosurveillance against infections and mammary tumors (24). In a recent study, we observed an increase in the in vitro IL-2 production and NK cell activity by splenocytes isolated from old male rats treated with deprenyl. Similar to the results observed in old rats, deprenyl treatment of rats with carcinogen-induced mammary tumors enhanced NK cell activity.

The increase in NK cell activity without an increase in the population of NK cells may be due to an increase in the production of several cytokines, especially, interferon-γ. Currently, we are measuring the concentrations of neurotransmitters in the medial basal hypothalamus by high peroformance liquid chromatography and Con A-induced IL-2 and IFN-g production by a bioassay and ELISA, respectively.

As stated in the Statement of Work, Experiment 1A has been completed with pending analyses of the levels of hormones in the serum and the neurotransmitters concentrations. Experiments 1C and 2 are being carried out and will be completed before August 1998.

Conclusions

Treatment of rats with carcinogen-induced mammary tumors markedly reduces the tumor growth and tumor number, and enhances NK cell activity. The actions of deprenyl on immune reactivity, especially, NK cell activity, is significant because of the involvement of NK cell activity in preventing metastasis of tumors.

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Figure Legends

Fig. 1. Effects of ip administration of 0, 0.25 mg, 2.5 mg, or 5 mg/Kg BW/day of deprenyl on the growth of tumors. Treatment with 2.5 mg and 5 mg of deprenyl for 13 weeks following the development of tumors significantly inhibited the growth of tumors.

Fig. 2. Effects of ip administration of 0, 0.25 mg, 2.5 mg, or 5 mg/Kg BW/day ofdeprenyl on the average number of tumors/rat. Treatment with 2.5 mg and 5 mg of deprenyl for 13 weeks significantly inhibited the tumor burden.

Fig. 3. Splenic NK cell activity in rats with carcinogen-induced mammary tumors after 13 weeks of treatment with deprenyl. In comparison to rats that received oil (vehicle for DMBA; without tumors), spleen cells from saline- and 0.25 mg deprenyl-treated rats showed reduced NK cell activity. Daily administration of 2.5 mg and 5.0 mg of deprenyl significantly enhanced NK cell activity.

Fig. 4. Con A-induced proliferation of spleen cells was unaltered in rats that were treated with saline and deprenyl but it was lower than that in rats that received oil alone.

Figure 1

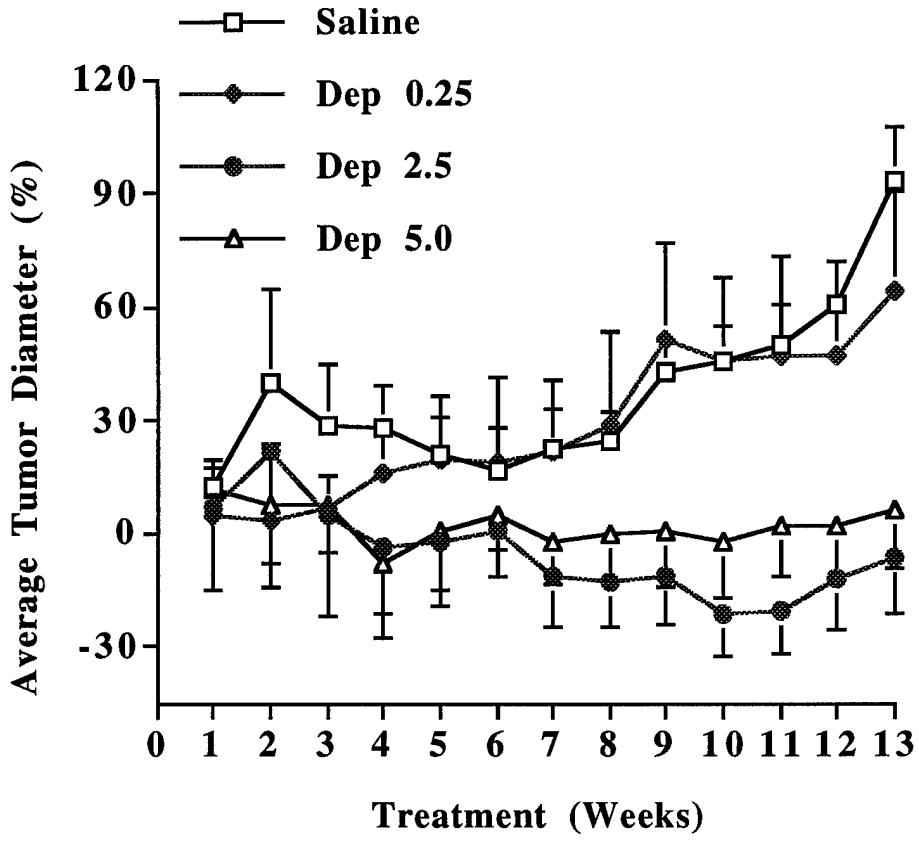
Saline
Dep 0.25
Dep 2.5
Dep 5.0
Average Tumor Diameter (%)
120
90
60
30
0
-30
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Treatment (Weeks)

Figure 2

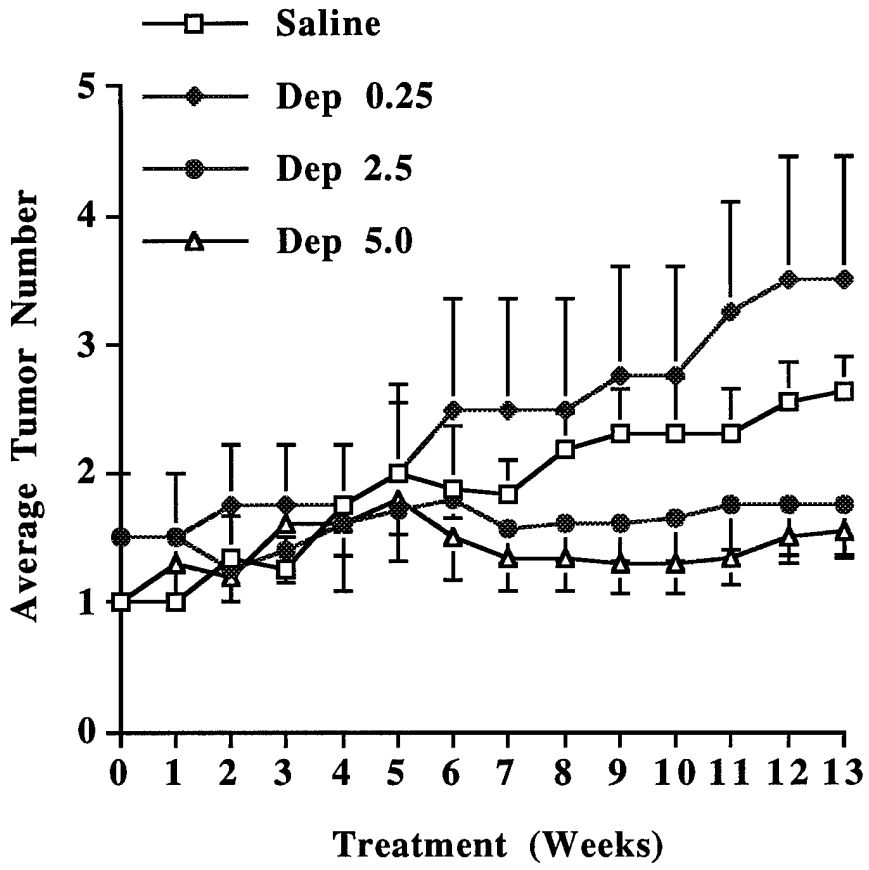
Saline
Dep 0.25
Dep 2.5
Dep 5.0
Average Tumor Number
0
1
2
3
4
5
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Treatment (Weeks)

Figure 3

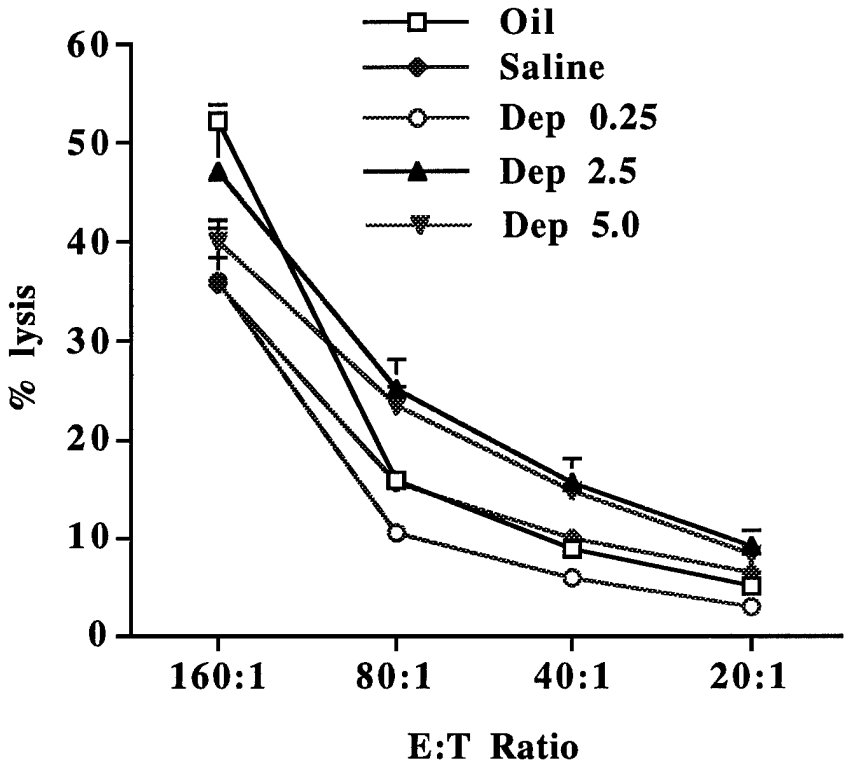
Oil
Saline
Dep 0.25
Dep 2.5
Dep 5.0
60
50
40
30
20
10
0
% lysis
160:1
80:1
40:1
20:1
E:T Ratio

Figure 4

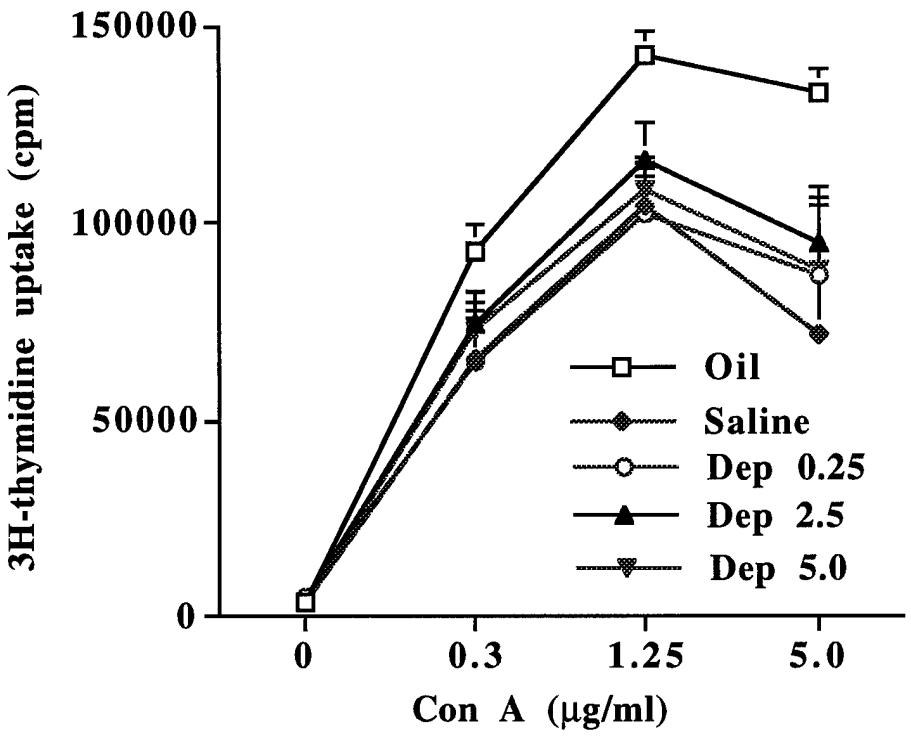
150000
100000
50000
0
3H-thymidine uptake (cpm)
0
0.3
1.25
5.0
Con A (µg/ml)
Oil
Saline
Dep 0.25
Dep 2.5
Dep 5.0

Table 1. Spleen lymphocyte population from rats with carcinogen-induced mammary tumors after 10 weeks of treatment with deprenyl.

Groups	% sIgM$^+$	% CD4$^+$	% CD8+	% NK+
Oil	36.7±1.2	44.9±1.0	15.7±0.8	3.9±0.4
Saline	40.1±1.6	43.06±1.4	12.0±0.9*	3.8±0.4
Dep 0.25	30.9±0.6	36.6±1.8	11.6±1.2*	3.7±0.3
Dep 2.5	35.7±1.8	39.0±2.1	11.8±0.7*	3.8±0.3
Dep 5.0	36.4±2.0	40.0±2.6	10.9±0.9*	3.1±0.7

Results are expressed as Mean±SEM

* Significantly different ($P<0.001$) from Oil